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Selective Quasispecies Transmission after Systemic or Mucosal Exposure of Macaques to Simian Immunodeficiency Virus

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Sexual transmission is the major cause of the AIDS epidemic. For the development of new antiviral and vaccine strategies, we therefore need to understand the mechanisms by which lentiviruses cross the mucosal barrier and the subsequent pathogenic consequences. For this purpose, experimental approaches are greatly facilitated by the development of relevant animal models. In this study, macaques were inoculated intravenously, intrarectally, or intravaginally with a pathogenic cell-free isolate of SIVmac251. Patterns of virological and immunological events significantly differed between vaginally (transient viremia, late seroconversion) and intravenously or intrarectally inoculated monkeys (persistent viremia and early seroconversion). Two weeks after infection, analysis of the *env* gene nucleotide sequences of proviruses recovered from PBMCs demonstrated that most of the differences were observed in the V1 loop. Three viral variants were specifically associated with vaginal transmission, whereas no such selection was evidenced after intravenous or intrarectal transmissions. These results are in favor of specific mechanisms associated with vaginal transmission, implicating viral envelope structure.

INTRODUCTION

The worldwide dramatic epidemic spread of human immunodeficiency virus (HIV) (Barré-Sinoussi *et al.,* 1983) infection, mainly attributed to sexual contacts, urges the need for designing new therapeutic and vaccine strategies, which depends on a better understanding of the complex mucosal infection mechanisms.

The investigation of human HIV mucosal transmission is limited by the poor access to biological samples within the very early moments of infection and the difficult identification of the source of transmitted viruses. The limited available data in humans tend, however, to confirm that although several viral variants may be identified in blood and genital fluids of transmitters, in the recipient the genotype and phenotype of transmitted viruses are homogeneous at the moment of seroconversion (Zhang *et al.,* 1993; Zhu *et al.,* 1993). This observation argues for a selection of viruses which cross the mucosal barrier and strongly suggests that specific mechanisms may account for sexual HIV transmission.

The *in vivo* approaches of these mechanisms are greatly facilitated by the development of particularly well-adapted animal models of human HIV infection; one of the most relevant is the experimental infection of macaques with pathogenic strains of SIVmac. This nonhuman primate lentivirus shares similar genomic organization and biological properties with HIV-1 and HIV-2 (Desrosiers, 1990). Interestingly, some SIV strains induce in macaques an immunodeficiency syndrome which strikingly reproduces human AIDS (Daniel *et al.,* 1985).

Using this animal model, we have designed an experiment in order to determine precisely the pathogenesis and the virological consequences of a possible selective pressure exerted by the mucosa. Macaques were exposed to the virus by intravenous, intrarectal, or intravaginal routes. Clinical, immunological, and virological outcomes were compared as well as the nucleotide *env* sequences of proviruses recovered from peripheral blood mononuclear cells (PBMCs) soon after inoculation.

RESULTS

Virological and immunological events following infection by intravenous, rectal, or vaginal routes

We compared the early pathogenic events following infection of macaques by the different inoculation routes. The kinetics of virological and immunological parameters subsequent to intravenous inoculation were previously characterized (Le Grand *et al.,* 1994) and found to be absolutely comparable to other reported experiments. The mean time (days postinoculation (pi) ± SD) of seroconversion, appearance of antigenemia, and cell-associated viremia of eight cynomolgus macaques inoculated by the intravenous (iv) route with 4 iv animal infectious doses at 50% (iv-AID_{50}) were, respectively, 18.87 ± 2.47, 11.37 ± 2.13, and 9.25 ± 2.18.

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TABLE 1

PCR Detection of SIV in PBMC and ELISA Detection of SIV-Specific IgG in Plasma from Macaques Inoculated by Vaginal (ivAG), Rectal (ir), and Intravenous (iv) Routes

		Results at pi week: SIV detection in PBMC/presence of SIV-specific antibodies in plasma										
Animals	Inoculation route	1	2	3	4	6	12	20	30	50	65	90
46683	ivAG	−/−	+/−	+/+	+/+	+/+	+/+	+/+	+/+			
46691	ivAG	+/−	+/−	+/+	+/+	+/+	+/+	+/+	+/+	−/+	+/+	+/+
46720	ivAG	−/−	−/−	−/−	−/−	−/−	+/+	−/+	+/+	−/+	−/+	−/+
J143	ivAG	−/−	+/−	+/+	+/+	+/+	+/+	+/+				
J482	ivAG	−/−	+/−	+/−	+/+	+/+	−/+	+/+				
374B	ivAG	−/−	+/−	−/−	−/−	+/−	−/+					
I872A	ivAG	+/−	+/−	+/−	+/−	+/+	−/−	−/−	+/+			
46681	ivAG	+/−	+/−	+/−	+/−	+/+	+/−	−/−	+/+	+/−	+/−	+/+
49098	ir	NT/−	+/−	+/NT	+/+	NT/+	+/+					
49105	ir	NT/−	+/−	+/NT	+/+	NT/+	+/+	NT/+	NT/+	NT/+	+/+	+/+
49100	ir	NT/−	+/−	+/NT	+/+	NT/+	+/+	NT/+	NT/+	NT/+	+/+	+/+
49091	ir	NT/−	+/−	+/NT	+/+	NT/+	+/+	NT/+	NT/+	NT/+	+/+	+/+
49088	ir	NT/−	+/−	+/NT	+/+	NT/+	−/+	NT/+	NT/+	NT/+	+/+	+/+
J849	iv	−/−	+/+	+/+	+/+	+/+	+/+					
J882	iv	−/−	+/+	+/+	+/+	+/+	+/+					
J644	iv	−/−	+/−	+/+	+/+	+/+	+/+					
I823A	iv	−/−	+/−	+/+	+/+	+/+	+/+					
46713	iv	+/−	+/+	+/+	+/+	+/+	+/+					
46880	iv	−/−	+/−	+/−	+/+	+/+	+/+					

Note. +, positive detection; −, negative detection; and NT, available sample not tested.

In macaques intrarectally inoculated with different virus stock dilutions, no major differences were observed when compared to intravenously inoculated animals (Table 1): the mean time (days pi ± SD) of seroconversion, appearance of antigenemia, and cell-associated virus load were, respectively, 22 ± 14 ($n = 9$), 14 ± 2.8 ($n = 11$), and 16 ± 2.5 ($n = 7$). However, the *in vivo* infectious titer of this virus stock was reduced approximately 200-fold after ir compared to iv inoculation.

Contrasting what we described for ir or iv inoculated animals, such a unique and reproducible pattern of infection was not observed in vaginally inoculated macaques. Interestingly, in half of these monkeys, atraumatic vaginal exposure to the pathogenic SIVmac251 resulted in a transient viremia accompanied by a weak and late antibody response (Table 1) (Le Grand *et al.,* 1995). Whereas persistently viremic macaques seroconverted within a delay similar to that observed for iv or ir infected animals (3 to 6 weeks following inoculation), transiently viremic macaques 374B and 46720 seroconverted at 12 weeks pi. No antigenemia was detected in these two macaques (Table 1). This particular infection pattern did not seem to be related to the inoculated dose or the presence of seminal plasma in the inoculum. By contrast, in our experiment, using this same virus stock at even low infectious doses (Le Grand *et al.,* 1994; Le Grand and Dormont, 1993), no such transient infection was observed in animals inoculated by the iv or ir route (Table 1).

Genetic characterization of transmitted viruses

In order to investigate whether the route of infection resulted in the transmission of specific SIVmac251 variants, we directly sequenced and compared the *env* gene, from variable regions V1 to V5, of proviruses obtained from PBMCs of 8, 5, and 6 infected animals inoculated by vaginal, rectal, or intravenous routes, respectively. Two of the vaginally inoculated monkeys exhibited a transient viremia (macaques 46720 and 374B). To avoid genetic variation under host immune pressure, day 14 postexposure samples were analyzed, except for a transiently viremic, vaginally inoculated monkey (46720) whose first detection of SIV provirus in PBMCs was obtained at 12 weeks pi. For all animals, later time point *env* sequences were also analyzed to confirm the predominance of the *env* sequences described below.

Among the proviruses identified at day 14 pi, we observed, accordingly with other reports (Almond *et al.,* 1992; Overbaugh *et al.,* 1991), that most of the envelope genotype diversity resided in the first (V1) and fourth (V4) variable regions (data not shown). However, in our study, whereas V1 nucleotide sequences displayed a mean of only 41% similarity with the consensus sequence of SIVmac251, substantially more sequence homology (93%) was found in V4. Therefore, we focused our work on the V1 loop, using a direct solid-phase sequencing method. This technique was previously shown to give results comparable to sequencing of multiple clones in deter-

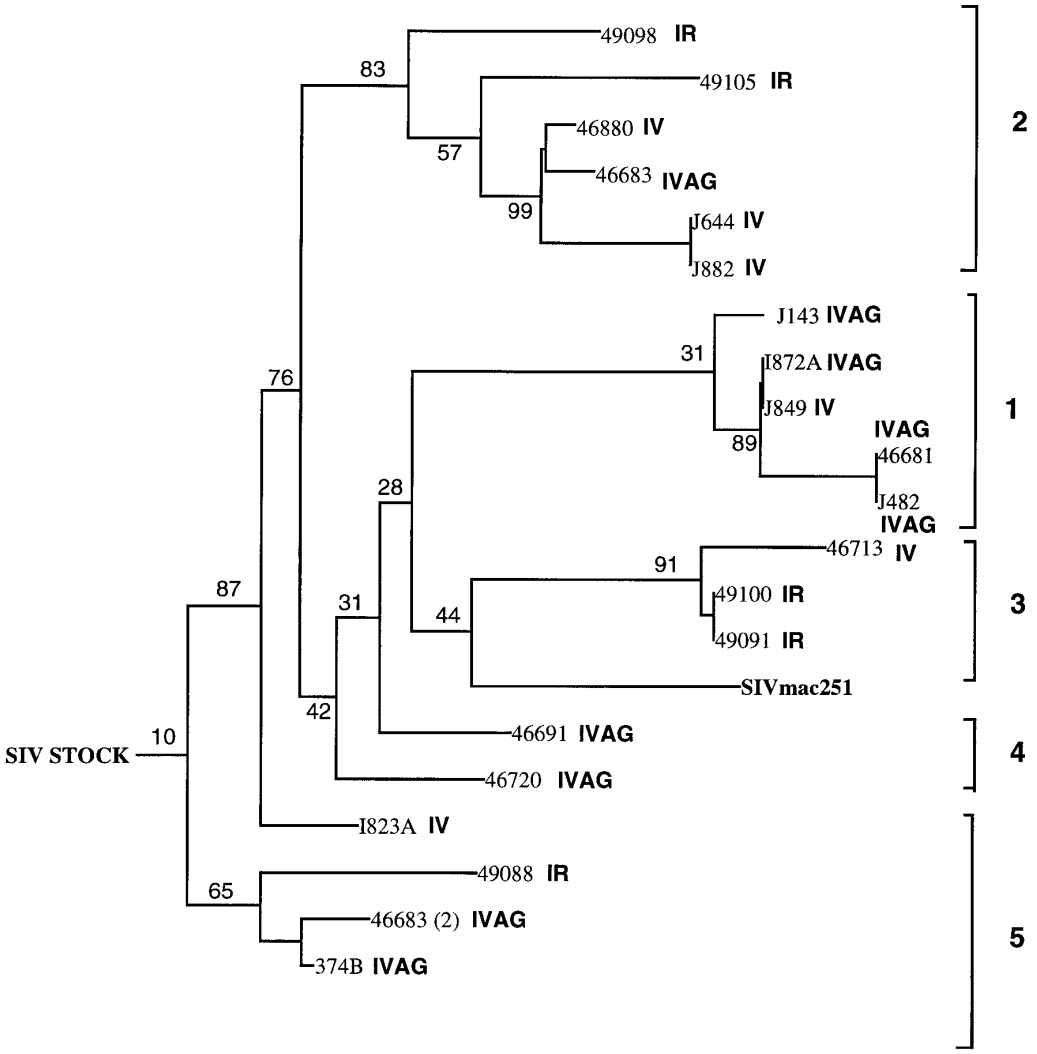

FIG. 1. Phylogenetic dendogram of the V1 (nucleotid 6948 to 7020) *env* DNA sequences recovered at day 14 pi (day 87 pi for macaque 46720) from PBMCs of macaques inoculated by intravenous (IV), intrarectal (IR), or intravaginal (IVAG) routes. Multiple DNA sequence alignments generated with CLUSTALW were resampled (bootstrap, 100 data sets) using SEQBOOT (PHYLIP). The consensus neighbor-joining dendogram (DNADIST; NEIGHBOR; CONSENSE, PHYLIP) is based on distances calculated by the two-parameter model of Kimura. The numbers at the forks indicate the number of times the group consisting of the species to the right of that fork occurred among the 100 trees. The numbers included at the right refer to the numeral of the group in which the macaque V1 peptide sequences are classified (see Fig. 2). SIV stock refers to the major nucleotide V1 sequence recovered from the inoculated virus stock. SIVmac251 refers to the consensus sequence of the SIVmac251. 46683 (2) refers to the V1 sequence recovered from macaque 46683 at 21 days pi.

mining the predominant nucleotide viral sequence in PBMCs isolated from infected patients (Leitner *et al.,* 1993; Scarlatti *et al.,* 1993). From our analysis, we deduced a phylogenetic tree showing that the sequences in V1 may be distributed among five major groups (Fig. 1). Interestingly, this analysis revealed a nonrandom distribution of the routes of infection among these five clusters; the deduced peptide sequences are presented in Fig. 2.

Group 1 contains the predominant V1 genotypes of four of the eight animals infected by the vaginal route and the sequence of only one of the six iv infected macaques. V1 peptide sequences from group 1 are characterized by the presence of a potential N-glycosylation site (N-X-T motif) which is not encountered in the consensus sequence of SIVmac251 and in the other subtypes presented here.

V1 sequences of the other vaginally infected macaques, including those which exhibited a transient viremia (46720 and 374B), were equally distributed among groups 4 and 5. The former includes also the sequence of the major V1 variant of monkey 46683 at 3 weeks pi, although its major genotype identified at day 14 pi was classified in group 2 as described below. Finally, the sequences of one ir (49088) and one iv inoculated monkey (I823A) were also found to be homologous to the group 5 consensus sequence. Although the major viral variant recovered from our virus stock displayed a V1 the

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Group 1
Consensus     TKSSTTT---TAPTTPNTTSTKPID
I872A         ........................  IVAG
J482          ........................  IVAG
46681         ........................  IVAG
J143          .......AST.............  IVAG
J849          ........................  IV

Group 2
Consensus     TKSSATAAPTSAPTSTTTSAKID
46880         ......T.........P......   IV
J882          ........................  IV
J644          ........................  IV
49098         ......T.......T.A.T-T..   IR
49105         ....T....S.ST......-A..   IR
46683         ..............P..-T..     IVAG

Group 3
Consensus     TKSSTTTTTTTAAPTAISD
46713         ...................       IV
49091         ...................       IR
49100         ...................       IR

Group 4
Consensus     TKSSXTTAXPXXTTTTASAXID
46720         ....A...A.TA.......K..    IVAG
46691         ....T...T.AS.......E..    IVAG

Group 5
Consensus     TKSSTTTASTTATPTPATATAD
374B          ......................    IVAG
49088         ...................ST.    IR
I823A         ..............A..SKI.     IV
46683 (2)     ...................S..    IVAG
SIV Stock     ......................

Unclassified
J482 (CVS)    TKSSTTTAAPTSVTTSID        IVAG
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FIG. 2. Deduced amino acid sequence alignments of the V1 region of SIV gp120 from PBMCs of intravaginally (IVAG), intrarectally (IR), or intravenously (IV) infected macaques at day 14 pi (day 87 pi for macaque 46720). Peptide sequences were clustered into five groups on the basis of the phylogenetic dendogram derived repartition (Fig. 1). Predominant viral variant recovered from the virus inoculum is indicated as SIV stock; 46683 (2) refers to the V1 sequence recovered from PBMCs of animal 46683 at day 21 pi; J482 (CVS) refers to predominant V1 sequence recovered from cervicovaginal secretions of macaque J482 at day 14 pi. Dots indicate sequence identity and dashes indicate deletions.

peptide sequence similar to the group 5 consensus sequence (Fig. 2), it was not the predominant genotype recovered from animals infected by the iv or ir routes.

Three of the six animals infected by the iv route, and two of the five macaques infected by the ir route, as well as only one vaginally inoculated monkey (46683), exhibited V1 sequences of genotype 2. It must be noted that, in this group, as observed in cluster 1, two series of sequences with different lengths were identified. One was characterized by the insertion of 3 bp encoding for an alanine at position 19 of the V1 loop and seemed to be associated only with iv transmission.

The third group of V1 genotypes is composed of three identical sequences from one intravenously and two intrarectally infected animals.

Distribution of viral variants in different tissues

In summary, we demonstrated that the virus variants identified early after infection from seven of the eight intravaginally infected macaques are grouped in three of the five distinct patterns of V1 genotypes. To verify that variants clustering with groups 2 and 3, which are not present in blood of the macaques infected by vaginal route, are sequestered in neither mucosal or lymphoid tissues, we analyzed and compared the number and sequence of viral variants detected in PBMCs, cervicovaginal secretions (CVS), or peripheral lymph node mononuclear cells (LNMCs) of three vaginally infected macaques displaying V1 sequences clustering in group 1 in blood at day 14 pi. For this purpose we performed a single-strand conformation polymorphism analysis of polymerase chain reaction products (PCR-SSCP). This method enables the separation of different single-stranded DNA on the basis of the composition of their nucleotide sequence by migration in a nondenaturing polyacrylamide gel (Hayashi, 1991; Hynes *et al.,* 1993). In all samples from macaques J143 and 46681, only one band, corresponding to the patterns of migration of sequences analogous to the group 1 consensus, was evidenced (Fig. 3). In samples from macaque J482, two bands, corresponding to two distinct viral variants, were identified. Those representing the group 1 sequence are located in the upper third of Fig. 3. This V1 variant, according with direct sequence data, seems to represent the major variant recovered from PBMCs and LNMCs from macaque J482. Conversely, in this same animal, PCR-SSCP analysis and sequence determination revealed that one other variant was predominant in CVS at day 14 pi. This V1 peptide sequence did not cluster in one of the five genotypes described above (Fig. 2).

Figure 4 shows the characteristic PCR-SSCP patterns of migration of the different V1 SIV variants recovered from monkeys included in this study. All identical DNA V1 sequences migrated to the same locus. To conclude, we never identified variants clustering in groups 2 or 3 in PBMCs of all vaginally infected animals except monkey 46683, in which at least two viral species, analogous to the group 2 and 5 sequences, were recovered (Fig. 4). Finally, using PCR-SSCP, we evidenced the presence of more than one viral variant in only two of the eight vaginally infected animals compared to four of the six iv infected animals and three of the five ir inoculated macaques (Fig. 4).

DISCUSSION

In this study, macaques were infected with the same cell-free pathogenic SIVmac251 stock by iv, ir, or vaginal atraumatic single inoculation. The parameters of infection we observed were almost similar in iv and ir infected animals, whereas an atypical pattern of infection was evidenced in some macaques inoculated by the vaginal

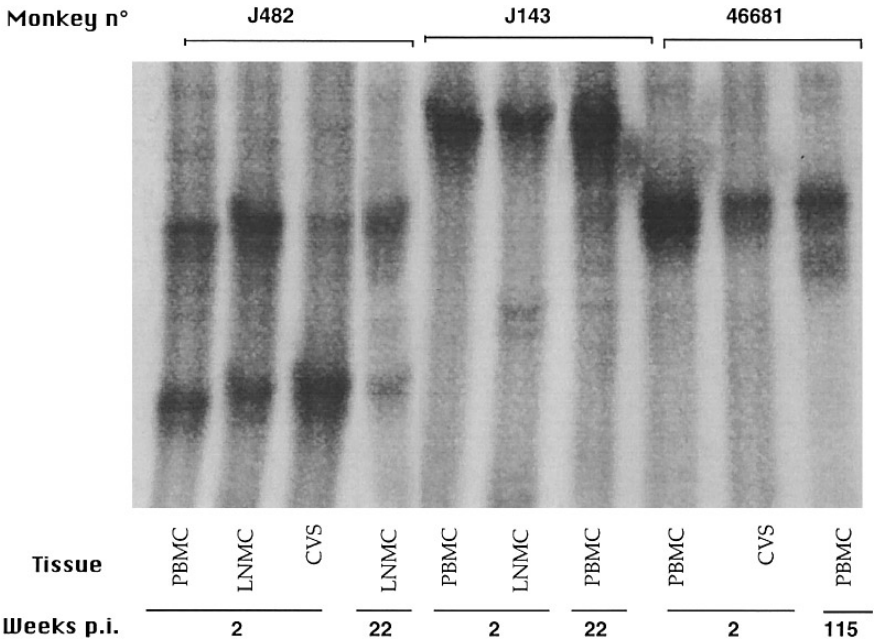

FIG. 3. PCR-SSCP analysis of DNA from cervicovaginal secretions (CVS), lymph node mononuclear cells (LNMCs), and PBMCs of intravaginally inoculated macaques J482, J143, and 46681. The V1 and flanking regions (nucleotides 6847 to 7133) of the SIV envelope from the indicated tissues of the three infected macaques were analyzed. The bands in the upper half of each V1 autoradiogram have been sequenced and correspond to the single-stranded conformation of the group 1 V1 sequences.

route, characterized by transient detection of proviral DNA and a weak and delayed antibody response. Such an infection pattern was previously reported by others after vaginal inoculation of macaques with SIV (Miller *et al.,* 1994). However, although transient viremia were observed also after ir inoculation of SIV (Pauza *et al.,* 1994), in our experiment, all the ir exposed monkeys exhibited a persistent viremia and an early and strong anti-viral immune response, even when low doses of virus were inoculated (Le Grand and Dormont, 1993). Nevertheless, this apparent discrepancy may be attributed to the origin of the viral stocks used. Indeed, the SIV prepared by Pauza *et al.* (1994) was grown on human cells which may have selected viral variants with different biological properties than primary isolates grown on monkey cells (Hirsch *et al.,* 1989). Our virus stock was obtained from a cell-free supernatant of SIVmac251-infected rhesus PBMCs and was never passaged on human PBMCs or cell lines. *In vitro,* this virus replicates on human T cell lines, simian and human PBMCs, as well as simian primary astrocytes and monocyte/macrophages (Guillemin *et al.,* 1996). Therefore, this SIVmac251 strain shares common properties with HIV primary isolates.

Several virological and/or host factors may account for the different infection profiles we observed between routes of experimental inoculation of SIV. One of the possible explanations is that specific SIV variants may be selected by the exposed mucosa. We therefore analyzed the *env* gene of transmitted viruses by a direct sequencing method. As previously reported (Leitner *et*

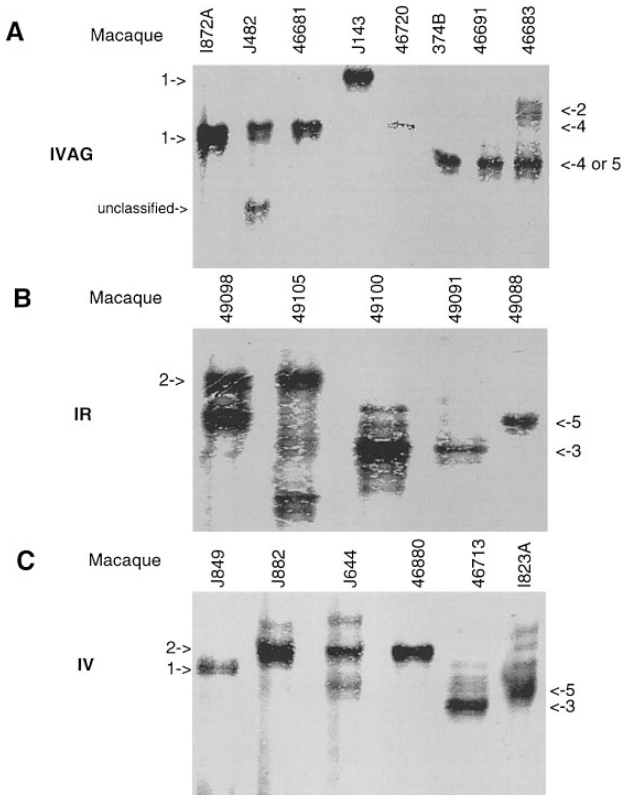

FIG. 4. PCR-SSCP analysis of V1 *env* DNA sequences obtained from PBMCs of (A) intravaginally (IVAG), (B) intrarectally (IR), or (C) intravenously (IV) inoculated macaques. The V1 and flanking regions (nucleotides 6847 to 7133) of the SIV envelope were analyzed. Respective locations of sequences analogous to the group 1 to 5 genotypes were confirmed by sequence analysis of the bands and are indicated by arrows.

al., 1993; Scarlatti *et al.,* 1993), this technique allows the determination of the predominant nucleotide viral sequence in virus-infected cells. Furthermore, in our experiment, sequence analysis of the major bands obtained from PCR-SSCP analysis revealed that these bands always corresponded to the major nucleotide sequence obtained from direct sequencing of animal samples.

We confirmed, as reported by others (Almond *et al.,* 1992, Overbaugh *et al.,* 1991), that variability in the envelope of SIVmac251 is mainly located in the first variable loop. In our animals, we identified five major transmitted genotypes with respect to their V1 nucleotide sequences. V1 sequences recovered from the majority of vaginally infected macaques were distinct from those recovered from the majority of the intravenously or intrarectally infected animals. SIV variants identified early after infection from seven of the eight vaginally infected macaques were grouped in three of the five distinct patterns of V1 genotypes, namely groups 1, 4, and 5. Based on PCR-SSCP analysis of LNMCs and CVS obtained from vaginally infected animals (Fig. 3), we may assume that the absence of sequences analogous to the variants clustering in groups 2 and 3 in PBMCs from vaginally infected animals (at the exception of macaque 46683) was not due to a restricted replication of these variants in the peripheral lymph nodes or mucosal tissues we tested. Therefore, this viral selection was likely to take place at the first entry step, the vaginal mucosa. However, we cannot exclude from our experiment that the absence of vaginal transmission of genotypes 2 and 3 in seven of the eight vaginally infected animals may reflect a limited viral replication of these variants in a tissular compartment that was not assessed.

In this experiment, we cannot distinguish between the proviruses obtained from transiently or persistently infected monkeys. Nevertheless, among vaginally infected animals, four of the six persistently viremic macaques exhibited a V1 sequence analogous to the group 1 genotypes. Interestingly, these variants are characterized by the presence of an additional potential N-glycosylation site. Overbaugh *et al.* (1992) already reported that during evolution of chronic SIV infection in macaques, new sites for N-linked glycosylation can appear in the V1 region, which may confer to these variants a selective advantage. We may therefore hypothesize that viruses displaying the group 1 V1 loop are favored, by virtue of their phenotype, in the efficient crossing of the vaginal mucosa compared with the other variants described here.

Finally, in this experiment, we cannot conclude, as previously suggested (Trivedi *et al.,* 1994), that there is a selection of particular viral genotypes after ir inoculation compared with the sequences obtained from iv-infected macaques. These results are concordant with the virological data we observed.

This study emphasizes the specific selective pressure exerted by the vaginal mucosa compared to the rectal mucosa and points out the fact that different transmission mechanisms may be involved for crossing through these two structurally distinguishable epithelium barriers (Fawcett, 1994).

Indeed, the vagina and exocervix exhibit a stratified epithelial cell layer and a submucosa which include different cell types that may be infected by HIV as Langerhans dendritic cells (LDC), macrophages, and $CD4^+$ T lymphocytes. Recent studies suggest that LDC may be actively implicated in experimental vaginal SIV transmission in macaques (Spira *et al.,* 1996) as well as sexual HIV transmission in humans (Soto-Ramirez *et al.,* 1996).

The rectum is covered by a simple epithelial monolayer (Fawcett, 1994); this cell composition does not include Langerhans cells (Lehner *et al.,* 1991). However, this mucosa is composed of several cell types which may be susceptible to HIV infection, including epithelial cells, through the galactosyl-ceramide receptor (Fantini *et al.,* 1993), and intraepithelial T lymphocytes, although no *in vivo* evidence of their infection has been described to date. Epithelial cells or the antigen-carrier M cells (Amerongen *et al.,* 1991; Bomsel, 1997) may also unselectively facilitate the transmission of viruses by transcytosis from the lumen to the *lamina propria,* where HIV may infect the resident macrophages and $CD4^+$ T cells before its release in lymphatic organs or bloodstream.

Our study provides, in a relevant animal model, clear evidence that different lentivirus genotypes may be distinguished by the route of transmission. These results emphasize also that the first variable loop of the envelope glycoprotein of SIVmac251 may be implicated in the interactions with the target cells the virus may encounter *in vivo* during sexual transmission. These cells, which therefore probably play a key role in the selection of infecting viral variants, may not be identical in the vaginal and in the rectal mucosa. As the transmitted viruses should be the target of vaccine strategies, further studies on their biological and antigenic properties could be of interest for the development of new vaccine approaches.

MATERIALS AND METHODS

Animals

Cynomolgus (*Macaca fascicularis*) or rhesus macaques (*Macaca mulata*) were imported from Mauritius island and were housed in single cages within level 3 biosafety facilities. All macaques were negative for SIV, STLV (simian T lymphotropic virus), Herpes-B, Filovirus, SRV-1, SRV-2, measles, hepatitis B-HB sAg, and hepatitis B-HB CAb before SIV inoculation. Animals were clinically examined periodically under ketamine chlorhydrate anesthesia (Rhone-Mérieux, Lyon, France). All experimental procedures were conducted according to institutional guidelines for animal care ("Journal Officiel des Communautés Européennes," L358, 18 December 1986).

Virus

The pathogenic SIVmac251 cell-free virus stock was kindly provided by Dr. A. M. Aubertin (Université Louis Pasteur, Strasbourg, France). This virus was grown on rhesus peripheral blood mononuclear cells (rhPBMCs) infected *in vitro* with a culture supernatant obtained from a coculture of rhPBMCs and spleen lymphocytes from a rhesus macaque infected with SIVmac251 (provided by Dr. R. C. Desrosiers, New England Regional Primate Research Center, MA). Using the method proposed by Spouge (1992), the *in vivo* titer of this stock was estimated to be 4×10^4 animal infectious doses at 50% per ml after iv inoculation of macaques (iv-AID_{50}), 2×10^2 per ml after atraumatic intrarectal exposure (ir-AID_{50}) (Le Grand and Dormont, 1993), and 67 per ml after atraumatic vaginal exposure (ivAG-AID_{50}).

Intravenous inoculation of macaques

Macaques were inoculated into the saphenous vein with 1 ml of a 10^{-4} dilution of the virus stock diluted in PBS, corresponding to 4 iv-AID_{50}.

Intravaginal inoculation of macaques

Four weeks before SIV inoculation, animals were treated with three intramuscular injections of 50 μg of estradiol benzoate (Laboratoires Roussel, Paris, France) at 48-h intervals. One milliliter of 10-fold dilutions of the virus stock was prepared in PBS. Before inoculation, final dilution was completed to 2 ml with PBS alone or undiluted human seminal plasma. The inoculation procedure involved deposition of either 2 ml of cell-free SIV diluted in PBS or 2 ml of cell-free SIV diluted in PBS containing 50% of human seminal plasma (hu-SP) into the vaginal vault using a soft-plastic tube originally intended for artificial insemination of dogs (Centravet, Lyon, France). Animals remained immobile for at least 15 min after inoculation. The dilutions of virus used were 5×10^{-2} corresponding to 3.3 ivAG-AID_{50} (macaques 46691, 46720, J143, J482, and 46683) and 5×10^{-3} corresponding to 0.33 ivAG-AID_{50} (macaques 46681, 374B, and I872A).

Intrarectal inoculation of macaques

Macaques were submitted to 48 h of hydric diet. One milliliter of 10-fold dilutions of the virus stock was prepared in PBS. Before inoculation, final dilution was completed to 3 ml with PBS. The inoculation procedure involved deposition of 3 ml of cell-free SIV diluted in PBS into the rectal vault using a smooth pediatric nasopharyngial tube. The virus dilutions used were 10^0 corresponding to 2×10^2 ir-AID_{50} (macaques 49098, 49105), 10^{-1} corresponding to 2×10^1 ir-AID_{50} (macaque 49100), and 10^{-2} corresponding to 2 ir-AID_{50} (macaques 49091, 49088). Macaques remained immobile for at least 15 min after inoculation.

Sample collection and PCR amplification

Blood samples were collected weekly for hematological, virological, and immunological analyses during 4 weeks after inoculation and monthly thereafter. Washes of the vaginal canal with 2 ml of sterile PBS were performed. Vaginal cells were separated from supernatant by 500*g* centrifugation and washed four times with PBS before being prepared for PCR assays. Fresh macaque PBMCs and LNMCs were isolated by Ficoll gradient separation (Eurobio, Les Ulis, France). DNA was extracted from PBMCs, LNMCs, and cells from vaginal washes (Le Grand *et al.,* 1994). For detection of SIVmac251 provirus, DNA was amplified using nested sets of primers specific for *env* and *gag* SIVmac251 genes (provided by the European Vaccine Against AIDS program, National Institute for Biological Standards and Control, Potters Bar, United Kingdom). The *env* outer and inner primer pairs were, respectively, 8125-N-AGTTACACTACTGGTGGCACCTCA, 9228-CCCCTCACAAGAGCGTGAGCTCAAG; and 8742-N-GGGGTATAGGCCAGTGTTCTCTTCCCCACC, 9228-CC-CCTCACAAGAGCGTGAGCTCAAG. The *gag* outer and inner primer pairs were 1386-N-GAAACTATGCCAAAAA-CAAGT, 2129-C-TAATCTAGCCTTCTGTCCTGG; and 1731-N-CCGTCAGGATCAGATATTGCAGGAACAACT, 2129-C-TAATCTAGCCTTCTGTCCTGG. A nested set of primers specific for *pol* was used to confirm the results obtained with the *env* and *gag* primers. The *pol* outer and inner primer pairs were 3988-N-ATTCCACTTACCAGTT-GAGAGG, 4623-C-CAGGTGTCTACTATCTGTCTGG; 4021-NACAGTTGGTGGACAGACTATTGG, 4373-C-GCCTG-CTCTTGATTCTGTAGG.

For sequence analysis, the *env* gene of SIVmac251 was amplified from 1 μg of the total genomic DNA, a previously described classical reaction mixture (Le Grand *et al.,* 1994), and an outer pair of primers: 6597-N-TCAGCTGCTTATCGCCATCGCCATCTTGC, 8208-C-AGAACCTGCCGTTGCGAAAACC; 35 main cycles were run (94°C for 45 s, 55°C for 2 min, and 72°C for 1 min). Two microliters of the first amplification mixture was used as template in a second round of amplification using the inner primer pairs spanning the region from V1 to V3: 6847-N-GTATGGCAACTCTTTGAGACCTCAATA, 7778-C-TCTCCTTCTGCAATTTGTCCACAT; and V3 to V5: 7410-N-TGGCTTTAATGGAACTAGAGCA, 8208-C-AGAAC-CTGCCGTTGCGAAAACC.

Detection of anti-SIV IgG in plasma by ELISA

Anti-SIV antibodies were detected in plasma as previously described (Le Grand *et al.,* 1994) using an HIV-2 detection assay (ELAVIA II kit; Sanofi Diagnostis Pasteur, Marnes-la-Coquette, France). As second antibody, the anti-human IgG provided by the manufacturer was replaced by peroxidase-labeled goat anti-monkey IgG (Organon, Teknika, the Netherlands).

Sequence analysis, alignments, and phylogenetic tree determination

A direct solid-phase sequence analysis was performed using the ABI PRISM Dye Terminator kit (Perkin–Elmer, CA) and the inner set of primers described above according to the manufacturer's procedures. Sequences were analyzed using the Sequence Navigator software (Applied Biosystems, CA); multiple alignments and phylogenetic tree were determined with CLUSTALW (Thompson *et al.,* 1994) and PHYLIP (Felsenstein, 1993) software.

Sequence analysis of the viral stock

Viral RNA was obtained from 1 ml of the viral stock by ultracentrifugation (100,000*g* for 5 min) and lysis with RNAble (Eurobio, Les Ulis, France). Extraction of RNA and reverse transcription were performed according to previously published protocols (Chéret *et al.,* 1996). Briefly, total RNA was subjected to first-strand cDNA synthesis for 1 h at 42°C in a mixture containing 0.25 M Tris–HCl, 0.375 M KCl, 15 mM $MgCl_2$, 30 U recombinant RNase inhibitor (Clontech, Palo Alto, CA), 30 mM each dNTP, 0.3 μg oligo(dT) (Sigma), and 150 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Grand Island, NY). After completion of first-strand synthesis, 10 μl of the reaction mixture was used for PCR with the *env* primers and sequenced as described above.

Single-strand conformation polymorphism analysis of PCR products (PCR-SSCP)

Products from the first-round PCR amplification mixture were used as template in a second round using the inner primer pair 6847-N and 7778-C spanning the region from V1 to V3 (25 pmol each), [α-^{33}P]dATP (18.5 MBq) (Amersham, USA), dNTP (10 mM each), *Taq* polymerase buffer, and 0.5 units of *Taq* polymerase (Appligen, Illkirch, France). To obtain a limited size of the DNA (286 bp) for a better migration, the products of the PCR were digested with 5 units of restriction enzyme *Rsa*I (16 h, 37°C), cleaving the *env* gene at positions 7133 and 7149. Samples were diluted 1:1 in stop solution (U.S. Biochemicals), denatured, and loaded into a nondenaturing polyacrylamide gel (Long-Ranger 6% (US Biochemicals), glycerol 5%) for migration during 6 h at 30 W and room temperature. The gel was dried and autoradiographied.

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